Screening and function studies in immunotoxicity testing

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SUMMARY

After a short introduction of various chemicals of environmental concern that have been shown to alter cell-mediated or humoral immune responses, a screening procedure is given to detect possible immunotoxic properties of chemicals. The different parameters in this screening programme include growth, weight and histology of lymphoid and endocrine organs, haematology, as well as serum immunoglobulin concentrations.

Next, different functional tests are discussed to assess the cell-mediated immunity, the humoral immunity, and the phagocytosis by macrophages in the rat. These tests should be performed when in a screening study an indication of immunotoxicity is found at a relevant dose level. The aim of functional assessment is to determine the functional significance of an effect on the immune response. As the data available at present clearly show that the developing organism is more at risk to the immunomodulating effects of different chemicals than the corresponding adult, functional assessment of immune effects should preferably be carried out after combined pre- and postnatal exposure.

INTRODUCTION

Soon after the identification of polychlorinated biphenyls (PCB) in tissues of fish and wildlife in the Netherlands (9), studies were initiated at the Institute of Veterinary Pharmacology and Toxicology to determine the toxicity of commercial PCB preparations. Following the observation of atrophy of lymphoid tissues in the spleen of chickens (24), and in the thymus (the central lymphoid organ of the cell-mediated immunity), spleen and lymph nodes of rabbits (21), the significance of the effect of PCB on the immune system was functionally assessed in the guinea pig. These studies (22) have clearly shown that PCB exposure suppresses the thymus-dependent humoral immunity (depressed serum antibody titers to tetanus toxoid and reduction of the number of tetanus antitoxin producing cells in lymph nodes) as well as the cell-mediated immunity (delayed-type hypersensitivity to tuberculin).

Prompted by the effects observed in PCB exposed animals, the related polybrominated biphenyls (PBB) were studied. In chickens, dietary exposure to PBB induced lymphoid depletion in the bursa of Fabricius (the central lymphoid organ of the humoral immunity) and spleen. Upon functional assessment, depressed prim-

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ary and secondary immune responses to tetanus toxoid were recorded in PBB exposed guinea pigs (23). In addition, lymphoid depletion was observed in the thymic cortex and in the splenic follicles and periarteriolar lymphocyte sheaths. There was increased interest in PBB with regards to its effect on the immune system after an accident occurred in 1973 in the state of Michigan, when a fire retardant consisting primarily of PBB was inadvertently substituted for magnesium oxide food supplement for livestock (4). Clinical observations of exposed cattle suggested that infections were often present.

With regard to immune effects, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) has been the most thoroughly studied halogenated aromatic hydrocarbon. TCDD is a highly toxic impurity that may be formed during the production of 2, 4, 5-trichlorophenol. Severe atrophy of the thymus has been reported as occurring in almost all laboratory animals exposed to sublethal doses of TCDD. Following the first report of TCDD induced thymic atrophy in the rat, depression of various cell-mediated immune parameters as well as thymus-dependent humoral immune responses was demonstrated in guinea pigs, mice, and rats (25, 31). Significant depression in the two latter species occurred only after exposure during the pre- and postnatal period.

Various organometallic compounds have been studied in respect of their potential immunotoxic properties. Of these different chemicals, dialkyltin compounds have been the most intensively studied, following the initial observation that di-n-octyl tin dichloride (DOTC) caused a severe thymic atrophy in the rat as result of a selective lymphocytotoxicity (14). Upon functional assessment, different parameters of both the cell-mediated and the thymus-dependent humoral immunity were suppressed by DOTC and the related compound di-n-butyl tin dichloride (DBTC). The immune suppression was most pronounced in rats that were exposed immediately after birth (15-17). Based on the results obtained with the different compounds listed above, the following methods are now used in the National Institute of Public Health to screen chemicals for possible immune effects, and to assess functionally the immune system of rats which are exposed to toxic chemicals or drugs.

Detailed information on immune effects of PCB, PBB, TCDD, dialkyl tins, and other compounds is given elsewhere (7, 12, 13, 20, 23, 28).

SCREENING TESTS TO DETECT IMMUNOTOXICITY

It is well established that the most profound effects of compounds which interfere with the immune response occur when the animal is confronted with the compound during the ontogenesis of the lymphoid system. A sensitive system to detect effects on the immune system is, therefore, a reproduction study which includes a thorough evaluation of the lymphoid system. However, this does not imply that studies to detect such effects should always be conducted in animals during the developmental phase of the immune system. For practical reasons, initial assessment could be done in a 3-week range-finding study or in a 3-month semichronic toxicity study.

During these experiments body weight gain and food intake are recorded. At the termination of the toxicity study, thymus, spleen, lymph nodes (popliteal and mesenteric nodes) are examined macroscopically, weighed, and processed for histopathological examination. For the determination of peripheral lymphocyte and monocyte numbers (as precursors of macrophages) total and differential leucocyte counts are carried out. The concentrations of the main serum immunoglobulin classes (e.g. IgM and IgG) can be measured by the enzyme-linked immunosorbent assay (ELISA) (27).

From these different parameters (weight gain, food intake, weight and histology of lymphoid organs, peripheral blood counts, serum IgM and IgG levels) a conclusion may be reached on whether the chemical has an effect on the immune system. Such an effect can be direct or indirect (secondary to an effect elsewhere, e.g. caused by malnutrition or an

altered endocrine balance). Especially an interaction of the chemical with the endocrine system which can indirectly cause an effect on the immune system should be considered, as various hormones (in particular glucocorticosteroids) do modify the immunological responses (20, 33). For this reason, pituitary gland, thyroid, adrenals, testes, and ovaries are also weighed an examined microscopically. If the effect on the immune system cannot be attributed to an indirect effect of which the functional significance is known, and the effect is a sensitive parameter, functional studies should be carried out.

Using the approach here described, a total of 17 different pesticides were recently screened in 3-week toxicity studies (M. J. van Logten and J. G. Vos, unpublished data). Of these compounds, three (triphenyl tin hydroxide, lead arsenate, and captan) had a significant effect on one of the various immune parameters as the most sensitive criterium. With these three chemicals, immune function tests are in progress in rats exposed after weaning as well as in rats exposed pre- and postnatally.

FUNCTION TESTS OF THE IMMUNE SYSTEM

Function studies of the immune system are necessary to gain an insight into the functional significance of the chemically induced effect on the immune system found in a routine study, in order to evaluate the potential risk of the chemical. Subtle effects on immune responses will be more easily detected if the animal is confronted with the chemical during the developmental phase of the lymphoid organs. If the chemical passes the placenta and is excreted in the milk, e.g. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (25) and hexachlorobenzene (30), pre- and postnatal maternal treatment is probably the most sensitive test system to detect an altered immune response. For chemicals which do not readily pass the placenta and are not readily excreted in the milk, e.g. di-n-butyl tin dichloride (16), pups can be treated postnatally by oral intubation. Function studies of the pups could be performed at the time of weaning or later.

The choice of function tests depends mainly on the effects seen in the routine toxicity study: if thymic atrophy is the main characteristic, the cell-mediated response should be studied first. In cases where the effect is primarily on serum immunoglobulins, one should test the capacity of the animal to generate a humoral immune response. Before starting function tests that allow of a separate study of the different phases of the immune response, one should obtain an insight into whether the overall response is impaired. For this purpose it is particularly useful to determine whether the resistance to infection is impaired by the chemical. *In vivo* and *in vitro* function tests will be successively described for the cell-mediated immunity, the humoral immune response to T-cell-dependent and T-cell-independent antigens, and the phagocytizing capacity of macrophages. Comprehensive information on these and other techniques is given elsewhere (1, 11, 32).

A. Cell-mediated Immunity

1. *Resistance to Listeria monocytogenes infection*

The resistance to *Listeria monocytogenes* is a combination of non-specific phagocytosis which inhibits or kills the growth of the organism during the first two days after infection, and cell-mediated immunity which starts operating from day 2 post-infection (3, 19). All phases of the cell-mediated immune response are involved in the acquired resistance to *Listeria*, including the participation of activated macrophages in the killing of *Listeria* in the effector phase. Animals are intravenously injected with *Listeria monocytogenes*. Criteria used to assess the resistance to this type of infection are mortality rates in control animals compared with those in the animals treated with the chemical, or bacterial enumeration in the spleen at days, 4, 6, or later after inoculation, when the cell mediated immunity interrupts the growth of the organism *in vivo*. Spleens of the injected animals are homogenized, and serial di-

lutions of each homogenate are plated to determine the viable counts of *Listeria* (19). Regarding the use of mortality rates as a criterium some doubts remain, since recent data indicate that athymic nude rats (unpublished data) or mice (6) are more resistant to *L. monocytogenes* infection than their thymus-bearing litter mates. Bacterial enumeration in spleen after a sublethal dose of *Listeria* therefore seems the best parameter by which to assess the acquired resistance to *Listeria*.

2. *Rejection of allografts*

In this technique, skin of inbred rats is transplanted to recipients that differ from each other at the major histocompatibility (Ag-B) locus or other important histocomptability loci (5). Subtle suppressive effects on cellular immunity may perhaps be missed, since considerable suppression is required to prolong the allograft rejection time by one or two days. A rapid method of grafting skin on tails is described by Bailey and Usama (2).

3. *Delayed-type hypersensitivity*

An important but not very sensitive test from measuring cell-mediated immunity is the delayed cutaneous hypersensitivity reaction to tuberculin (8). In this test, animals are preferably sensitized with a subcutaneous injection, in the foot pad, of an oil suspension containing killed *Mycobacterium tuberculosis* (H37Ra adjuvant), intradermally challenged with tuberculin PPD during the second and third week after sensitization, and measuring, with calipers, the diameter and thickness of the skin reaction after 24 and 48 h. as. parameter of cell-mediated immunity. An alternative and probably more objective and sensitive method for measuring tuberculin skin hypersensitivity in rats (10) is based on the radioactive labelling, with tritium thymidine, of bone marrow precursors of monocytes, which cells accumulate in delayed-type hypersensitivity reactions and are measured by liquid scintillation counting of a biopsy specimen of the challenged ear. A disadvantage of this test is the *in vivo* use of a radioactive label. As an alternative, the thickness of the delayed reaction in the ear can be measured with calipers. Instead of assaying the hypersensitivity to tuberculin, similar delayed reactions can be elicited by the using of a protein antigen (ovalbumin). Rats are sensitized in the foot pads with a mixture of ovalbumin in H37Ra adjuvant, and intradermal skin test are performed with ovalbumin. The advantage of using ovalbumin is that this protein also induces high antibody titers (cf. Section B1), whereas the optimum immunization dose of ovalbumin for measuring delayed-type hypersensitivity as well as the humoral immune response is the same (26). Thus, parameters of both the cell-mediated and the humoral immunity can be assayed simultaneously in the same animal.

4. *Transformation of lymphocytes by PHA and Con A*

The ability of lymphocytes to transform and incorporate labelled thymidine in DNA following nonspecific *in vitro* stimulation with the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) is a measure of T-cell function, as shown by the absence of a response of spleen cells from athymic nude rats (29), and permits of separate analysis of the adaptive phase of the immune response. In contrast to activation by specific antigens, these mitogens activate a relatively high percentage of lymphocytes, probably representing a polyclonal response. A practical and labour-saving microplate culture system is described by Thorpe and Knight (18). These authors also provide data for optimizing culture conditions and labelling technique.

B. Humoral Immunity

1. *Thymus-dependent antibody synthesis to tetanus toxoid or ovalbumin*

The antibody response to tetanus toxoid and ovalbumin is thymus-dependent in the rat (i.e. it needs the cooperation of so-called T-helper cells), as athymic nude rats do not generate an IgM or IgG antibody response to these antigens (29).

Rats are immunized with tetanus toxoid intravenously or in the footpad with a mixture of H37Ra adjuvant and ovalbumin.

As discussed in Section A3 the advantage of using ovalbumin as antigen is that both the humoral and the cell-mediated immunity can be assayed in the same animal. In addition, ovalbumin is highly immunogenic in the rat and generates, besides IgM and IgG, also antibodies of the IgE class. Serum antibody titers are determined by the enzyme-linked immunosorbent assay (ELISA) as described elsewhere for tetanus toxoid (27) and ovalbumin (26).

2. *Thymus-independent antibody synthesis to LPS*

The antibody response to *Escherichia coli* lipopolysaccharide (LPS) does not require the cooperation by T-helper cells, since a similar antibody response is achieved in the athymic nude rat as compared with thymus-bearing litter mates (29). Additional evidence that the humoral immune response to LPS *E. coli* is a thymus-independent phenomenon comes from the observation that immunological memory did not develop after the primary immunization with LPS. LPS is poorly immunogenic in the rat, but by means of ELISA it is possible to determine reasonable serum titers (27).

3. *Transformation of lymphocytes by LPS*

E. coli lipopolysaccharide (LPS) is a B-cell mitogen (T-cell independent) in the rat, as a normal response is seen in spleen cells from athymic nude rats (29). Information on LPS stimulation of rat lymphocytes is scarce, which may be due to the fact that the response of cell suspensions of lymphoid organs of the rat to this mitogen is poor (34).

C. Phagocytosis by Macrophages

Clearance of Listeria monocytogenes

As discussed in section A1, the resistance to *Listeria monocytogenes* is a combination of nonspecific phagocytosis and cell-mediated immunity. Nonspecific phagocytosis and killing can be measured shortly (day 1 and 2) after the intravenous inoculation of *Listeria* organisms, at a time when the cell-mediated immunity is not yet developed. Spleens of the injected animals are homogenized, and serial dilutions of each homogenate are plated to determine the viable counts of *Listeria*, which is a measure of the phagocytic and killing activity of macrophages.

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